Activation of p38 mitogen-activated protein kinase in spinal cord contributes to chronic constriction injury-induced neuropathic pain

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Abstract: The present study aimed to investigate the role of spinal p38 mitogen-activated protein kinase (p38 MAPK) activation in chronic constriction injury (CCI) of the sciatic nerve induced neuropathic pain. CCI model was produced by loosely ligating the left sciatic nerve proximal to the sciatica’s trifurcation with 4-0 silk thread in male Sprague-Dawley rat. SB203580, a specific inhibitor of the p38 MAPK, was intrathecally administered on day 5 post-CCI. Thermal and mechanical nociceptive thresholds were assessed with the paw withdrawal latency (PWL) to radiant heat and the paw withdrawal threshold (PWT) to von Frey filaments respectively. The protein levels of the phosphorylated p38 MAPK (p-p38 MAPK) and phosphorylated cAMP response element binding protein (pCREB) were assessed by Western blot analysis. The results showed that CCI significantly increased the expressions of cytosolic and nuclear p-p38 MAPK in the spinal cord. Intrathecal administration of SB203580 dose-dependently reversed the established mechanical allodynia and thermal hyperalgesia induced by CCI. Correlated with behavior results, SB203580 dose-dependently inhibited the CCI-induced increase of the expressions of cytosolic and nuclear p-p38 MAPK and nuclear pCREB in the spinal cord. Taken together, these findings suggest that the activation of p38 MAPK pathway contributes to the development of neuropathic pain induced by CCI, and that the function of p-p38 MAPK may partly be accomplished via the CREB-dependent gene expression.

Key words: p38 mitogen-activated protein kinase; cAMP response element binding protein; neuropathic pain; spinal cord
Varying etiologies-induced peripheral nerve injury may produce chronic neuropathic pain states characterized by hyperalgesia, allodynia and spontaneous pain [1]. To date, there is no effective treatment for relieving neuropathic pain and its mechanisms are unclear. Recently, synaptic plasticity of the spinal cord neurons induced by long-lasting nociceptive stimuli (also called central sensitization) is under intensive investigation [2-4]. It is believed that the central sensitization is mediated by a complex biochemical cascade initiated by the activation of primary afferent fibers. A large number of studies have shown that several protein kinases, for example, protein kinase A (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase II (CaMK II), play a role in the induction, development and maintenance of central sensitization [5-7].

Recently, some studies have reported that p38 mitogen-activated protein kinase (p38 MAPK), a member of MAPK family, contributes to pain hypersensitivity and central sensitization. Acute noxious stimuli, such as formalin, capsaicin, and spinal nerve ligation (SNL), induced p38 MAPK phosphorylation in the spinal dorsal horn neurons and p38 MAPK inhibitor reduced the acute pain behavior after subcutaneous formalin, capsaicin injection, or SNL [8-10]. However, the roles of p38 MAPK in the development of chronic neuropathic pain are unknown.

The transcription factor cAMP response element binding protein (CREB), one of the important downstream substrates of p38 MAPK, is critical for activity-dependent gene expression. CREB has been proposed to contribute to central sensitization associated with persistent pain states [11-13]. In a generally accepted view, NMDA activation-induced Ca$^{2+}$ influx triggers an early phase of CREB phosphorylation and a persistent phase of CREB phosphorylation mediated by a delayed MAPK signal cascade [14]. The latter is more important during the development and maintenance of chronic pain. Therefore, we hypothesize that peripheral nerve injury can activate the p38 MAPK in the spinal cord, and then the p38 MAPK translates to nuclear and phosphorylates CREB. The latter mediates the roles of p38 MAPK in the development of chronic neuropathic pain. In the present study, we used the chronic constriction injury (CCI) of the sciatic nerve model to investigate (1) whether activation of p38 MAPK in the spinal cord is involved in the maintenance of chronic neuropathic pain; (2) the effect of activation of p38 MAPK on phospho-CREB (pCREB) expression.

1 MATERIALS AND METHODS

1.1 Animals
Sprague-Dawley rats (200–250 g) provided by the Experimental Animal Center of Xuzhou Medical College, were kept under a 12 h/12 h light-dark cycle regime, with free access to food and water. All experiments were approved by the Animal Care and Use Committee at the Xuzhou Medical College and were in accordance with the college’s guidelines for the care and use of laboratory animals.

1.2 Implantation of intrathecal catheter
For intrathecal drug administration, rats were implanted with catheters as described by Yaksh and Rudy [15]. In brief, under anesthesia with pentobarbital sodium (40 mg/kg, i.p.), rats were fixed, the occipital muscles were bluntly separated, and then the cisternal membrane was exposed. Polyethylene catheters (PE-10) were inserted via an incision in the cisterna magna, and advanced 7.0–7.5 cm caudally to the level of the lumbar enlargement. Correct intrathecal placement was confirmed by injection of 10 µl 2% lidocaine through the catheter. The catheter was judged to be intrathecal if paralysis and dragging of bilateral hind limbs occurred within 30 s of this injection. Animals with signs of motor dysfunction were excluded from the experiment. The rats were housed individually after surgery and allowed to recover 5–7 d before the CCI test.

1.3 Chronic constriction injury
Chronic constriction injury of the sciatic nerve was performed as previously described by Bennett and Xie [16]. Briefly, under anesthesia with isoflurane, the left sciatic nerves of rats were exposed at the level of the middle of the thigh, and then four ligatures (4-0, silk thread) were tied loosely around proximal to the sciatica’s trifurcation at 1.0-mm intervals. Sham surgery was done by exposing the left sciatic nerve without ligatin.

1.4 Drug administration
Intrathecal drug administration was accomplished using a microinjection syringe connected to the intrathecal catheter in awake, briefly restrained rats. The injection was performed manually over a 30 s period in a single injection volume of 10 µl followed by a flush with 10 µl physiologica saline.

Different doses (0.1, 0.5, 2.5, 5 µg) of 4-(4-fluorophenyl)-2- (4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) (Biomol Research Laboratories Inc., USA) [dissolved in 2% dimethylsulfoxide (DMSO)], a p38 MAPK inhibitor, were intrathecally injected on day 5 post-CCI. DMSO was injected as control. Behavioral tests were performed 1 h pre-drug and 0.5, 3, 6, 12, and 24 h post-drug.
1.5 Behavioral studies

Mechanical allodynia was assessed by using von Frey filaments (Stoelting, Wood Dale, IL). Animals were placed in individual plastic boxes (20 cm × 25 cm × 15 cm) on a metal mesh floor and allowed to acclimate for 30 min. The filaments were presented, in ascending order of strength, perpendicular to the plantar surface with sufficient force to cause slight bending against the paw and held for 6–8 s. Brisk withdrawal or paw flinching were considered as positive responses. The paw withdrawal threshold (PWT) was determined by sequentially increasing and decreasing the stimulus strength (the “up-and-down” method), and the data were analyzed using the nonparametric method of Dixon, as described by Chaplan et al.[17].

Thermal hyperalgesia was assessed with the paw withdrawal latency (PWL) to radiant heat according to the protocol of Hargreaves et al.[18]. Rats were placed in clear plastic cages on an elevated glass plate and allowed to acclimate for 30 min before test. A high intensity light beam was focused onto the plantar surface of the hindpaw through the glass plate. The nociceptive endpoints in the radiant heat test were the characteristic lifting or licking of the hindpaw, and the time to the endpoint was considered the PWL. To avoid tissue damage, a cut-off time of 30 s was used. There were 3 trials per rat and 5 min intervals between trials.

To study the effect of SB203580 on the rat’s motor function, motor functions were evaluated by the observation of placing/stepping reflexes and righting reflexes and the rat was conducted 5 min before the assessment of nociceptive responses. Eight animals per group were used for behavior tests.

1.6 Western blot

Rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.). The lumbar sacral spinal cords of the rats were extracted and stored in liquid nitrogen. Tissue samples were homogenized in lysis buffer A (in mmol/L, pH 7.9): HEPES 10, NaVO₄ 1, MgCl₂ 1.5, KCl 10, NaF 50, edetic acid (EDTA) 0.1, egtazic acid (EGTA) 0.1, phenylmethylsulfonyl fluoride (PMSF) 0.5, dithiothreitol (DTT) 1 and 0.02% protease inhibitor cocktail. After the addition of 90 µl NP-40 (10%), the homogenates were vortexed for 30 s and then centrifuged at 800 g for 15 min at 4 °C. The supernatants were used for Western blot analysis as cytosolic proteins. The nuclear pellets were resuspended in buffer B (in mmol/L, pH 7.9): HEPES 20, NaCl 420, MgCl₂ 1.5, EDTA 1, EGTA 1, PMSF 0.5, DTT 1, 20 % glycerol, and 0.02% protease inhibitor cocktail. The homogenates were incubated for 30 min in ice-cold water with constant agitation and then centrifuged at 13 000 g for 15 min at 4 °C to separate the nuclear proteins. Protein concentrations were determined using the Bradford method [19] and the protein samples were stored at –80 °C.

Protein samples were dissolved in 4 × sample buffer (in mmol/L: Tris-HCl 250, Sucrose 200, DTT 300, 0.01% Coomassie brilliant blue-G, and 8% sodium dodecyl sulfate, pH 6.8), and denatured at 95 °C for 5 min, then the equivalent amounts of proteins (40 µg) were separated by using 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The membranes were incubated overnight at 4 °C with the following primary antibodies: mouse monoclonal anti-p-p38 MAPK antibody (1:400, Santa Cruz, Biotechnology, USA) or rabbit polyclonal anti-Ser133-pCREB (1:400, Santa Cruz, Biotechnology, USA). The membranes were extensively washed with Tris-Buffered Saline Tween-20 (TBST) and incubated for 1 h with the secondary antibody conjugated with alkaline phosphatase (AP) at room temperature. The immune complexes were detected by using a NBT/BCIP assay kit (Promega, Shanghai, China). The scanned images were imported into Adobe Photoshop software (Adobe, California, USA). Scanning densitometry was used for semiquantitative analysis of the data.

1.7 Experimental groups

To study the effect of intrathecal injection of SB203580 on CCI-induced thermal hyperalgesia and mechanical allodynia, rats were divided into six groups as following: rats without any treatment + intrathecal injection of SB203580 5 µg (naive + SB5 group), CCI + intrathecal injection of DMSO (CCI + DMSO group), CCI + intrathecal injection of SB203580 0.1, 0.5, 2.5 or 5 µg (CCI + SB0.1 group, CCI + SB0.5 group, CCI + SB2.5 group, CCI + SB5 group). To study the time course of p-p38 MAPK expression in the spinal cord of CCI rats, rats were divided into five groups: naive group, sham group, CCI 5 d group, CCI 10 d group and CCI 15 d group. To explore the effect of intrathecal injection of SB203580 on CCI-induced p-p38 MAPK and p-CREB expression, the following six groups were included: sham group, CCI group, CCI + DMSO group, CCI + SB0.1 group, CCI + SB0.5 group and CCI + SB5 group. The rat spinal cord was extracted 6 h post-drug.

1.8 Statistical analysis

All data are expressed as mean±SD. Statistical analysis was carried out using one-way ANOVA or Student’s t-test. P<0.05 was considered statistically significant.
2 RESULTS

2.1 CCI increased the expression of p-p38 MAPK in the spinal cord of rats

CCI, and not sham surgery, produced significant mechanical allodynia and thermal hyperalgesia. The time courses of PWT and PWL were presented in Fig. 1A and B. Compared with naive or sham group, the protein levels of both cytosolic and nuclear p-p38 MAPK were increased in the spinal cord of CCI rats. The expression of p-p38 MAPK, especially in the nuclear fraction, reached a peak level on day 10 post-CCI. The time course of changes of p-p38 MAPK, at least in part, correlated significantly with CCI-induced mechanical allodynia and thermal hyperalgesia (Fig. 1C).

2.2 Intrathecal injection of SB203580 reversed CCI-

Fig.1. Time courses of CCI-induced mechanical allodynia (A) and thermal hyperalgesia (B) and the p38 MAPK (38 kDa) activation in the spinal cord (C) in rats. All data were expressed as mean±SD. n = 8, *P<0.05, **P<0.01 vs d 0 in behavior test; n = 4, *P<0.05, **P< 0.01 vs naive group in Western blot test.

Fig.2. Intrathecal injection of SB203580 reversed CCI-induced mechanical allodynia (A) and thermal hyperalgesia (B) and the spinal p38 MAPK activation (C). All data were expressed as mean±SD. n = 8, *P<0.05, **P<0.01 vs CCI+DMSO in behavior test; n = 4, *P<0.05, **P<0.01 vs CCI in Western blot test.
### Intra-thecal injection of SB203580 inhibited the CCI-induced increase of nuclear pCREB expression in the spinal cord

The Western blot results revealed that, compared with sham group, CCI significantly increased the expression of nuclear pCREB in the spinal cord. Intrathecal administration of SB203580 dose-dependently inhibited the increase of nuclear pCREB expression in the spinal cord (Fig. 3).

![Fig. 3. Effect of intrathecal injection of SB203580 on the CCI-induced pCREB (43 kDa) expression in rats. All data were expressed as mean±SD. n= 4, **P<0.01 vs sham group; *P<0.05, ***P<0.01 vs CCI group.](image)

### DISCUSSION

In the present experiment, we demonstrated, for the first time, that CCI could induce long-lasting p38 MAPK phosphorylation in the spinal cord, and that the time course of p38 MAPK activation, at least in part, significantly correlated with behavioral hyperalgesia. Furthermore, intrathecal injection of SB203580, a p38 MAPK inhibitor, remarkably reversed CCI-induced mechanical allodynia and thermal hyperalgesia and activation of p38 MAPK in a dose-dependent manner. Another finding in the present study was that intrathecal injection of SB203580 also dose-dependently inhibited the CCI-induced increase of pCREB expression in the spinal cord. These results suggest that activation of p38 MAPK pathway contributes to the development of neuropathic pain induced by CCI, and that the function of p-p38 MAPK might partly be accomplished via the CREB-dependent gene expression.

The mechanisms underlying p-p38 MAPK mediated neuropathic pain are unknown. Nerve fibers produce abnormal ectopic excitability at or near the site of nerve ligation after CCI. The local persistent abnormal excitability of sensory nerve can spread to distant parts of the peripheral including the peripheral nerve bodies in the dorsal root ganglion (DRG) and central nervous system. Repeated or prolonged noxious stimulations and the persistent abnormal input following nerve injury increase the release of nociceptive neurotransmitters, such as glutamate, ATP, substance P, calcitonin gene-related peptide (CGRP), and BDNF in the central terminals of primary sensory afferents and then activate NMDA and NK receptors in the spinal cord. Calcium influx through NMDA receptor triggers Ras-raf/MAPK cascades responses. Several reports demonstrated that the phosphorylation of p38 MAPK was increased by Ca\(^{2+}\) influx [20-22].

Phospho-p38 MAPK could translocate from cytoplasm into nuclear and in turn phosphorylate transcriptional factor CREB on Ser-133. In agreement with this view, we found that CCI significantly increased the nuclear fraction p-p38 MAPK expression. Moreover, the time course of CCI-induced CREB activation in the spinal cord was in correspondence with that of p38 MAPK activation and intrathecal injection of SB203580 remarkably inhibited the increase of pCREB expression. Previous studies demonstrated that NGF activated the p38 MAPK and its downstream effector, MAPK-activated protein kinase 2 (MAPKAP kinase 2), resulting in phosphorylation of CREB on Ser-133. Inhibition of the p38/MAPKAP kinase 2 pathway partially blocked NGF-induced CREB Ser-133 phosphorylation and contributed to the activation of immediate early genes [23]. In addition, inhibition of p38 MAPK with SB203580 significantly attenuated ATP receptor activation-induced CREB phosphorylation [24]. A lot of studies have shown that ATP receptor activation in the spinal cord microglia was essential for pain hypersensitivity after nerve
injury. Phosphorylation of CREB on Ser-133 recruits the CREB binding protein, CBP, to the complex and promotes transcription of downstream genes. Many “pain genes”, which may contribute to central sensitization, are activated by CREB, including the immediate early gene c-fos, BDNF, CGRP, the alpha subunit of CaMK II and neurokinin 1 receptor[25-27]. A considerable amount of evidence have indicated that CREB-dependent gene expression was required for long-term changes in synaptic plasticity induced by various nociceptive stimuli[11-13, 28-30].

The specific target proteins of p38 MAPK pathway are generally unknown. The recent studies have indicated that several signal molecules may be involved in the roles of p38 MAPK activation on nociceptive information modulation. Obata et al. reported that the p38 MAPK inhibitor reduced SNL-induced thermal hyperalgesia and the increase in BDNF and TRPV1 mRNA in dorsal root ganglion[31]. Microglia are a source of multiple cytokines, including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α, which contribute to the development of pathological pain. Microglia also express cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), which lead to the synthesis of prostaglandin (PG) and nitric oxide (NO). Both PG and NO can enhance pain sensitivity. The p38 MAPK activation appears to regulate the expression of cytokines, COX-2, and iNOS via transcriptional, post-transcriptional (mRNA stability), and translational regulation[32-34]. In fact, it has been reported that selective inhibition of p38 MAPK suppressed IL-1β-induced COX-2 expression and PGE2 production, suggesting that IL-1β-induced PGE2 synthesis is up-regulated by the activation of p38 MAPK pathway[35].

In summary, our study indicates that the activation of p38 MAPK pathway contributes to the development of neuropathic pain induced by CCI, and that the function of p-p38 MAPK may partly be accomplished via the CREB-dependent gene expression.

REFERENCES


